REMARKS

Applicants respectfully request entry of the amendments and remarks from Applicant's October 8, 2008 Reply with this request for continued examination.

Claims 2, 5, 6, 8, 9, 11, 12, 14-38 and 53-65 are pending in this application. Claims 21, 22, 25 and 28-34 were withdrawn from consideration. Claims 1, 3-4, 7, 10, 13 and 39-52 were previously canceled.

THE REJECTIONS

Claim rejection under 35 U.S.C. § 103(a)

Claims 2, 5, 6, 8, 9, 11, 12, 14, 23, 24, 26, 27, 35-38, 53, 56 and 57

In the November 14, 2008 Advisory Action, the Examiner maintained the rejection of claims 2, 5, 6, 8, 9, 11, 12, 14, 23, 24, 26, 27, 35-38, 53, 56 and 57 under 35 U.S.C. § 103(a) as being obvious over the teachings of Kelly in view of Kuberasampath and Lefer. The Examiner states that Kelly teaches that materials designed to inhibit neutrophil-endothelial interactions and prevent the accumulation of neutrophils in the kidney are useful for the treatment of acute renal failure (ARF) in humans. The Examiner contends that it would have been obvious to one of ordinary skill in the art to use OP-1, a material designed to inhibit neutrophil-endothelial interactions, as taught by Kuberasampath and Lefer, to prevent the accumulation of neutrophils in the kidney. The Examiner states that although Kelly exemplifies these teachings by

blocking ICAM-mediated neutrophil adhesion, it does not diminish the generality of its teachings regarding the nature of materials that will be useful to block neutrophil adhesion. The Examiner states that although a large number of adhesion molecules may be sequentially activated during the multi-step process of transendothelial neutrophil migration, Kelly demonstrates that blocking a single molecule is sufficient. The Examiner further contends that in view of Kelly's teachings and Kuberasampath's and Lefer's teachings the OP-1 is effective in blocking neutrophil-endothelial interactions, one of ordinary skill in the art would have a reasonable expectation that OP-1 would block neutrophil accumulation in the kidney. Finally, the Examiner asserts that one of ordinary skill in the art would be motivated to combine the teachings of Kelly with Kuberasampath and Lefer in order to treat ARF because Kelly teaches that blocking neutrophil-endothelial cell interactions leads to an improvement in standard markers of renal function during ARF and Kuberasampath and Lefer teach that OP-1 is effective in blocking neutrophil-endothelial interactions. Applicants traverse.

Applicants respectfully maintain that the skilled worker would have no motivation to combine the teachings of Kelly with those of Kuberasampath and Lefer for the following reasons. First, Kelly indicates that inhibiting ICAM-1 may protect against renal ischemic injury, but it is not clear that ICAM-1 was acting entirely via potentiation of neutrophil-endothelial interactions. Kelly states that the depletion of neutrophils also protected against renal ischemic injury but that this is only indirect

evidence that the role of ICAM-1 in renal injury is linked to neutrophil-endothelial interactions.

Applicants point out that a proper determination of *prima facie* obviousness requires that the Examiner consider *all teachings in the analogous prior art* and what the combined teachings would have suggested to the skilled artisan. The MPEP states that

> [w]here the teachings of two or more prior art references conflict, the examiner must weigh the power of each reference to suggest solutions to one of ordinary skill in the art, considering the degree to which one reference might accurately discredit another. See MPEP § 2143.01 (II) citing *In re Young*, 927 F.2d 588, 18 USPQ2d 1089 (Fed. Cir. 1991).

As evidence of the state of the art at the priority date of the application, applicants submit herewith Sligh et al., "Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1," PNAS, 905:8529-8533, (1993) ("Sligh"), a copy of which is attached herein as Appendix A. Sligh demonstrated that ICAM-1 deficiency results in inhibition of mixed lymphocyte reaction (MLR) (see Figure 5). Sligh discloses that MLR inhibition is consistent with an important costimulatory role for ICAM-1 beyond its role in migration, suggesting ICAM-1 has other activities that may be responsible for the renal injury protection observed in Kelly.

As further evidence of the uncertainty of the relationship of ICAM-1 to neutrophil-endothelial interactions, applicants submit herewith Issekutz et al., "Role of ICAM-1 and

ICAM-2 and alternate CD11/CD18 ligands in neutrophil transendothelial migration," Journal of Leukocyte Biology, 65:117-126, (1999) ("Issekutz"), a copy of which is attached herein as Appendix B. Issekutz demonstrated that ICAM-1 inhibition alone was not sufficient to inhibit neutrophil transendothelial migration (TEM). Issekutz discloses that different mechanisms of TEM function in concert and that ICAM-1 has a redundant role in neutrophil-endothelial interactions, suggesting some other activity may be responsible for the renal injury protection observed in Kelly. Issekutz also states that strategies for regulating leukocyte migration in vivo, designed to block the ligands on vascular endothelium for CD11/CD18 integrins, will likely be very difficult to develop due to multiple and redundant interactions. Therefore, one of ordinary skill in the art would not conclude that any inhibition of neutrophil-endothelial interactions would necessarily confer protection against ARF.

Second, the disclosures of Kuberasampath and Lefer fail to remedy the deficiencies of Kelly. Kuberasampath and Lefer disclose that OP-1 inhibits neutrophil adherence to the endothelium. However, there is no teaching or suggesting that such inhibition would result in treatment of ARF. Given the difficulties and uncertainties in designing blockers of neutrophil-endothelial interactions as described in Issekutz, one of skill in the art would not have reasonably expected that OP-1 would protect against ARF based simply on its role in neutrophil adherence to the endothelium. One of skill in the art would not have reasonably expected that the effects of inhibiting an ICAM-1 mediated

neutrophil-endothelial cell interaction would be predictive of the effects of inhibiting any other adhesion molecules.

For all the above reasons, the skilled worker would not be motivated to combine the teaching of Kelly, Kuberasampath and Lefer. Accordingly, applicants respectfully request this rejection be withdrawn.

Claims 2, 15-20, 53-55 and 58-65

In the November 14, 2008 Advisory Action, the Examiner maintained the rejection of claims 2, 15-20, 53-55 and 58-65 under 35 U.S.C. § 103(a) as being obvious over the teaching of Kelly in view of Kuberasampath and Lefer and further in view of Anderson") and Brady. The Examiner states that Kelly in view of Kuberasampath and Lefer teach or suggest the use of an OP/BMP renal therapeutic agent to improve a standard marker of renal function in ARF as discussed above. Applicants traverse.

Applicants respectfully maintain that at least for the reasons described above, nothing in Kelly, Kuberasampath and Lefer teaches or suggests the use of an OP/BMP renal therapeutic agent to improve a standard marker of renal function in ARF. Applicants submit that nothing in Anderson and Brady, either alone or in combination with any of the other documents, remedies this deficiency. Anderson discloses impaired cardiac output is a major cause of acute deterioration in renal function. Brady discloses that low cardiac output is a major cause of pre-renal acute renal failure. However, nothing in Anderson or Brady teaches or suggests

a role for an OP/BMP renal therapeutic agent in regulating ICAM-1 and improving a standard marker of renal function in acute renal failure (ARF). Accordingly, applicants respectfully request that the Examiner withdraw this rejection.

CONCLUSION

In view of the foregoing remarks, applicants request that the Examiner reconsider and withdraw all outstanding rejections and allow the pending claims.

The Examiner is invited to telephone applicants' representatives regarding any matter that may be handled by telephone to expedite allowance of the pending claims.

Respectfully submitted,

/Ryan Murphey/

Karen Mangasarian (Reg. No. 43,772)
Attorney for Applicants Under 37 CFR 1.34
Ryan Murphey (Reg. No. 61,156)
Agent for Applicants Under 37 CFR 1.34

ROPES & GRAY LLP (Customer No. 1473)
1211 Avenue of the Americas
New York, New York 10036-8704
(212) 596-9000

Appendix A

Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8529–8533, September 1993
Genetics

Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1

(cell adhesion/immune deficiency/homologous recombination/gene targeting/lymphocyte interactions)

JAMES E. SLIGH, JR.*, CHRISTIE M. BALLANTYNE†, SUSAN S. RICH‡, HAL K. HAWKINS§, C. WAYNE SMITH¶, ALLAN BRADLEY*,**, AND ARTHUR L. BEAUDET*¶‖**

*Institute for Molecular Genetics, and Departments of †Internal Medicine, ‡Microbiology and Immunology, §Pathology, and ¶Pediatrics, Baylor College of Medicine and **Howard Hughes Medical Institute, Houston, TX 77030

Communicated by Joseph L. Goldstein, May 24, 1993

ABSTRACT Gene targeting was used to produce mice deficient in intercellular adhesion molecule 1 (ICAM-1) or CD54, an immunoglobulin-like cell adhesion molecule that binds β_2 integrins. Homozygous deficient animals develop normally, are fertile, and have a moderate granulocytosis. The nature of the mutation, RNA analysis, and immunostaining are consistent with complete loss of surface expression of ICAM-1. Deficient mice exhibit prominent abnormalities of inflammatory responses including impaired neutrophil emigration in response to chemical peritonitis and decreased contact hypersensitivity to 2,4-dinitrofluorobenzene. Mutant cells provided negligible stimulation in the mixed lymphocyte reaction, although they proliferated normally as responder cells. These mutant animals will be extremely valuable for examining the role of ICAM-1 and its counterreceptors in inflammatory disease processes and atherosclerosis.

Intercellular adhesion molecule 1 (ICAM-1) or CD54, a cell-surface protein with five immunoglobulin-like domains, plays an important role in transendothelial migration of leukocytes (1–3) through its expression on vascular endothelium and binding to β_2 leukocyte integrins (4, 5). The β_2 integrins are heterodimers composed of a common β subunit encoded by the *CD18* gene, combined with one of three α chains: CD11a for lymphocyte function-associated antigen 1 (LFA-1), CD11b for Mac-1, and CD11c for p150,95. Immunoglobulin domains 1 and 2 of ICAM-1 are involved in binding to LFA-1 (6), while immunoglobulin domain 3 of ICAM-1 mediates binding to Mac-1 (7). ICAM-1 is upregulated through synthesis of new protein *in vivo* and/or *in vitro* in response to inflammatory cytokines (8, 9), phorbol esters (10), or lipopolysaccharide (11).

Transendothelial migration of leukocytes begins with leukocyte rolling, which is largely dependent on selectins (12), followed by activation of integrins, firm attachment to endothelium, and migration across the endothelial surface (13). Integrin binding to ICAM-1 is particularly important for firm attachment and migration across the endothelium (13); for example, migration of human neutrophils through a monolayer of umbilical vein endothelium was inhibited >85% by anti-ICAM-1 monoclonal antibodies (mAbs) (1). Blocking antibodies to ICAM-1 inhibit migration of neutrophils *in vivo* in response to inflammation in the lung (14) and myocardium (15).

ICAM-1 is also implicated in various immune responses (16). Using allogeneic mouse or human cells *in vitro*, there is profound inhibition of the mixed lymphocyte reaction (MLR) by mAbs to ICAM-1 (11). ICAM-1 as well as other adhesion molecules can provide costimulatory signals for B-cell (17) and T-cell activation *in vitro* (18, 19). mAbs to ICAM-1 resulted in 50% reduction in contact hypersensitivity in mice (20).

No animals with mutations in ICAM-1 are reported. We sought to test the role of ICAM-1 in intact animals by disrupting the gene in murine embryonic stem (ES) cells.

MATERIALS AND METHODS

Targeting Construct and Generation of Mutant Mice. To prepare the targeting construct, a 5.5-kb segment of the *Icam-1* gene (21) containing exons 4–7 was cloned into pBluescript II KS(−) (Stratagene). A neomycin-resistance gene (*neo*) cassette containing a short version of the RNA polymerase II promoter and the bovine growth hormone polyadenylylation signal (22) was inserted at the *Hin*dIII site in exon 5.

The AB1 ES cell line was electroporated as described (23) with the construct after digestion with *Bgl* I for use as a replacement vector. Selection was performed with G418 (300 μg total weight per ml) for 9 days at which time individual G418-resistant colonies were picked. Screening for targeted recombination was performed either by Southern blotting using a microextraction procedure (24) or by PCR analysis of a portion of the colony using a primer contained within the *neo* cassette (oligonucleotide 2, 5′-GGACAGGTCGGTCTTGACAA-3′) paired with an outside primer (oligonucleotide 1, 5′-TGTGGGTAAAGGAAGGGACT-3′) located in the 5′ flanking region. PCR screening was performed by recovery of individual colonies in 20 μl of trypsin solution; half of the cell suspension was added to a final volume of 20 μl containing 50 μg of proteinase K per ml, 1.7 μM SDS, and 10 mM Tris at pH 8.0 and was incubated at 55°C for 1 hr. PCR was carried out for 35 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 1.5 min as described (25).

Cells confirmed by Southern blotting to carry the replacement mutation were injected into day 3.5 C57BL/6J blastocysts and transferred into foster mothers (26). Chimeric males were mated with C57BL/6J females, and germ-line transmission of the mutation was documented by Southern blotting of tail DNA by using *Bam*HI digestion and the 5′ flanking probe.

Reverse Transcription–PCR (RT–PCR). RNA was isolated from fresh tissues by using guanidinium isothiocyanate (27). Single-stranded cDNA was prepared with the SuperScript Moloney murine leukemia virus reverse transcriptase kit (Bethesda Research Laboratories). The cDNA product was

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Abbreviations: ICAM-1, intercellular adhesion molecule 1; MLR, mixed lymphocyte reaction; LFA-1, lymphocyte function-associated antigen 1; ES cells, embryonic stem cells; DNFB, 2,4-dinitrofluorobenzene; RT–PCR, reverse transcription–PCR; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody.

‖To whom reprint requests should be addressed.

amplified by using primers within exon 4 (oligonucleotide 3, bases 668–684: 5′-CTTCCAGCTACCATCCC-3′), exon 7 (oligonucleotide 4, antisense to bases 2244–2228: 5′-AGAACCACTGCTAGTCC-3′), and exon 5 (oligonucleotide 5, bases 1049–1064: 5′-GTTCTTCTGAGCGGCGT-3′); base pair numbering is as reported (28). PCR conditions were as described above with 10 cycles of 94°C for 30 sec, 65°C for 30 sec (decreasing 1°C each cycle), and 72°C for 30 sec followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec.

Peripheral Blood Analysis and Chemical Peritonitis. Peripheral blood cell counts and chemical peritonitis were performed as described (29).

Contact Hypersensitivity. Contact hypersensitivity was elicited in the mice by using the 2,4-dinitrofluorobenzene (DNFB) sensitization protocol exactly as described (30). Ear thickness was measured 24, 48, and 72 hr after DNFB challenge and the change in ear thickness (T) was calculated as ΔT.

Mixed Lymphocyte Reaction (MLR). MLRs were performed by mixing spleen cells from BALB/c animals with spleen cells harvested from wild-type or mutant hybrid (129/Sv × C57BL/6J) animals. Spleen cells were isolated and cultured in supplemented Dulbecco's modified Eagle's medium (sDMEM), and T lymphocytes were prepared as described (31). Stimulator cells were irradiated with 1500 R (1 R = 0.258 mC/kg). Enriched T cells (0.6 to 8 × 10^6 per ml) and irradiated stimulator spleen cells (4 or 8 × 10^6 per ml) were cocultured for 5 days in 96-well flat-bottom microtiter plates in sDMEM and then incubated for the final 18 hr with [^{3}H]thymidine (32).

Histology and Immunohistochemistry. Animals were sacrificed with or without a 6-hr-previous i.p. injection with 50 μg of lipopolysaccharide from *Salmonella typhosa* (Difco) dissolved in 0.5 ml of H_2O. Immunohistochemistry was performed by fixing freshly isolated tissues in 10% formalin in phosphate buffer. Fluorescent staining for ICAM-1 was performed by using the fluorescein isothiocyanate (FITC)-conjugated 3E2 antibody (20).

Flow Cytometry. Indirect immunofluorescent analysis was performed on leukocytes with a EPICS Profile flow cytometer (Coulter) using FITC-conjugated 3E2 antibody to ICAM-1 and with the phycoerythrin-conjugated RA3-6B2 antibody to CD45R (B220) (both from PharMingen). Two-color staining was performed by incubating 5 × 10^5 splenocytes in 100 μl containing 1 μg of each specific antibody or isotype-matched antibodies (PharMingen) on ice for 20 min.

RESULTS

Mutating the *Icam-1* Gene by Homologous Recombination. Homologous recombination was used to introduce a *neo* expression cassette within exon 5 of the *Icam-1* gene as described (Fig. 1*A*). Recombinant colonies were identified among the G418-resistant clones by screening with either PCR or Southern blotting. The mean frequency of homologous recombination was 1 in 70 G418-resistant colonies.

Targeted clones were injected into blastocysts, giving rise to male chimeric mice that transmitted the mutated *Icam-1* gene to the germ line of offspring as documented by Southern blotting. Heterozygous (+/−) mutant animals were intercrossed, and homozygous mutant animals were born in the expected ratio representing 26.5% of 260 progeny tested. The homozygous mutant (−/−) animals gained weight normally, were fertile, and did not demonstrate any obvious phenotype or susceptibility to infection when maintained with sterilized cages and food. Homozygous mutant inbred 129/Sv animals also were obtained and showed viability similar to animals of hybrid (129/Sv × C57BL/6J) background; all data shown below are for hybrid animals.

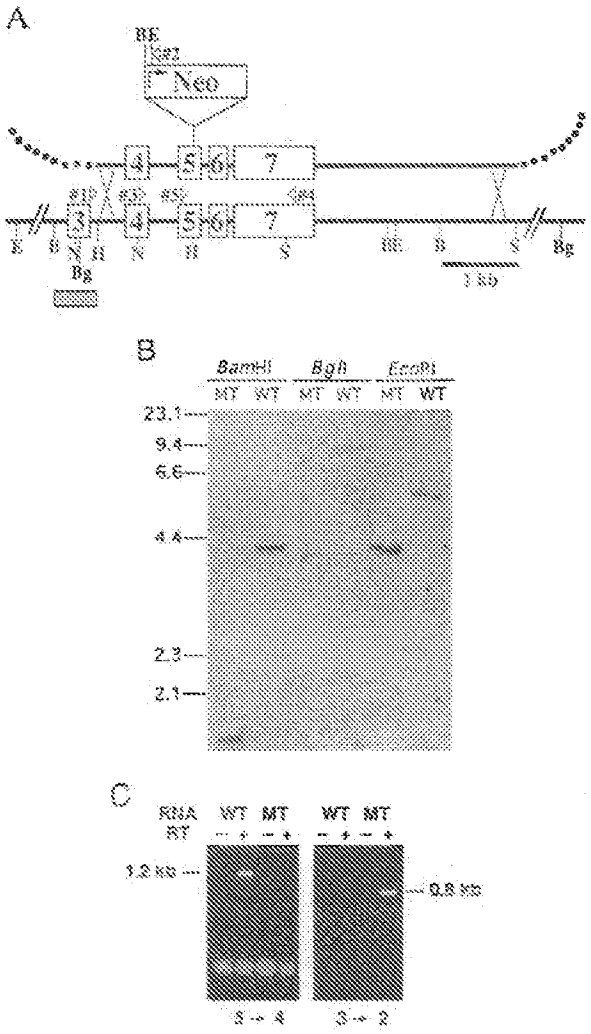

FIG. 1. Preparation and analysis of targeted mutation. (*A*) The targeting vector (upper diagram) is drawn to scale with exons numbered within boxes, the location of the *neo* cassette indicated, a solid line for mouse genomic DNA, and a dotted line for plasmid sequence. The lower diagram is of the mouse genomic DNA to be targeted. The location of oligonucleotide primers for PCR and RT–PCR are designated 1 to 5. The location of a flanking probe is indicated by the shaded box below. B, *Bam*HI; Bg, *Bgl* I; E, *Eco*RI; H, *Hin*dIII; N, *Nhe* I; and S, *Sal* I. (*B*) Southern blot analysis was performed with the flanking probe and genomic DNA isolated from tails of wild-type (lanes WT) and homozygous mutant (lanes MT) mice. (*C*) RT–PCR was performed by using RNA isolated from lung. RNA was prepared from wild-type (lanes WT) or mutant (lanes MT) tissue, and RT was omitted or added as indicated. Oligonucleotide primer pairs were 4 and 5 (*Left*) and 2 and 3 (*Right*).

Southern blotting analysis comparing DNA from wild-type and homozygous mutant animals was consistent with the expected mutation (Fig. 1*B*). Use of an upstream flanking DNA probe and digestion with *Bam*HI or *Eco*RI, both of which cut within the *neo* cassette, revealed the smaller 1.8- or 4.3-kb DNA fragments, respectively, consistent with the predicted replacement mutation. Digestion with Bgl I, which cuts outside the sequences contained in the vector, demonstrated a larger fragment that is increased in size by the 1.3-kb length of the inserted *neo* cassette.

***Icam-1* Mutation Eliminates Cell-Surface Expression.** To analyze expression of mutant transcripts, RNA was isolated from lung for RT–PCR. Using primers 5 and 4, which flank the mutation site from upstream of the *neo* cassette in exon

5 to exon 7, the expected 1.2-kb product is obtained with RNA from wild-type animals but not from mutant animals (Fig. 1*C*). Similar results were obtained with the primer in exon 5 and a primer in exon 6 (not shown), confirming the absence of normal mRNA in homozygous mutant animals. A sense-oriented primer in exon 4 (primer 3) and an antisense primer in the *neo* coding region (primer 2) were used to detect a transcript that might arise if exon 4 were spliced to the mutated exon 5, and the 0.8-kb product predicted for such a transcript was detected with RNA from mutant but not from wild-type animals (Fig. 1*C*). It is possible that a truncated form of ICAM-1 could be produced ending with aberrant sequence at the *Hin*dIII site in exon 5, but such a product would not have a transmembrane domain.

Histopathologic examination performed on three male and three female mice ranging in age from 8 weeks to 8 months did not reveal any abnormalities in tissue architecture. Thymus, spleen, liver, brain, eye, heart, skeletal muscle, bone, testis, ovary, skin, pancreas, stomach, small and large intestine, mesenteric and superior cervical lymph node, submandibular gland, adrenal gland, kidney, seminal vesicle, uterus, and lung were examined. Immunofluorescent staining of lung was performed by using the 3E2 antibody directed against ICAM-1. ICAM-1 is known to be expressed abundantly on alveolar capillary endothelium and on the luminal surface of type 1 alveolar epithelial cells in the mouse (C. Doerschuk, personal communication). Sections of lung taken from animals 6 hr after i.p. injection of lipopolysaccharide demonstrated abundant expression of ICAM-1 in cells surrounding airspaces in wild-type animals, but no immunostaining was visible with homozygous mutant animals (Fig. 2).

As a quantitative assessment of ICAM-1 expression, flow cytometric analysis of B lymphocytes was performed by using double-color analysis of cells stained with the 3E2 mAb directed against murine ICAM-1 and anti-B220 directed against the B-cell form of CD45R (Fig. 3). Flow cytometric analysis showed substantial expression of ICAM-1 on activated B lymphocytes from wild-type animals (mean fluorescence 4.2), but there was no significant expression of ICAM-1 on B lymphocytes from homozygous mutant animals (mean fluorescence 0.22 compared with 0.14 for the isotype-matched control antibody). The RT–PCR data, the immunohistochemistry, and the flow cytometry are all consistent with the complete loss of surface expression of ICAM-1.

Mutant Animals Show Granulocytosis but Normal Lymphocyte Populations. Although animals appeared phenotypically normal, subtle abnormalities could be identified in the resting state. Since ICAM-1 is strongly implicated in neutrophil emigration, peripheral blood neutrophil counts were performed. The neutrophil count ± SD was $1.0 \pm 0.5 \times 10^3$ per μl for wild-type animals ($n = 12$) and was increased to $4.1 \pm 1.6 \times 10^3$ per μl for homozygous mutant animals ($n = 14$) at 2–4 months of age ($P = 5 \times 10^{-6}$). As an additional evaluation of the cellular phenotype of the mutants, analyses of cell populations in spleen and thymus were performed by flow cytometry. No differences were found for wild-type and mutant animals for populations of $CD11a^+$, $ICAM\text{-}2^+$, $CD3^+$, $CD45R^+$ (B220), $CD4^+$, and $CD8^+$ cells in the spleen. Similarly no differences were found for thymic T-cell subsets of $CD4^+$ $CD8^-$, $CD4^-$ $CD8^+$, or $CD4^+$ $CD8^+$ cells.

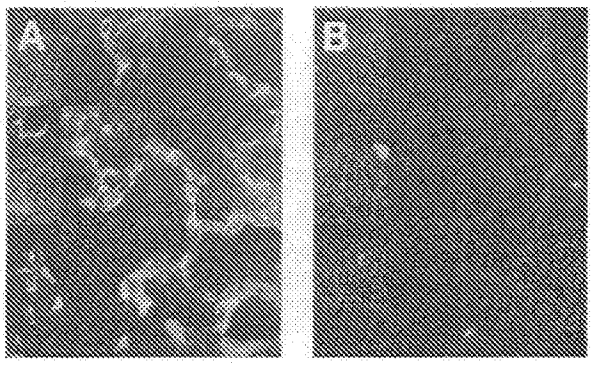

FIG. 2. Immunohistofluorescent staining of lung from wild-type and homozygous mutant mice. Wild-type (*A*) and mutant (*B*) animals were sacrificed 6 hr after i.p. injection of 50 μg of lipopolysaccharide. Lungs were stained with the 3E2 monoclonal antibody to mouse ICAM-1. Only weak autofluorescence is seen in mutant tissue.

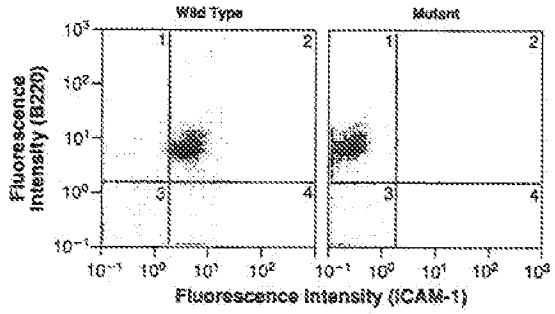

FIG. 3. Expression of ICAM-1 on B lymphocytes from wild-type (*Left*) and mutant (*Right*) mice. Cells were isolated from spleen and stimulated in culture for 15 hr with 200 ng of ionomycin per ml and 1 ng of phorbol 12-myristate 13-acetate per ml. Cells were stained with phycoerythrin-labeled anti-B220 (RA3-6B2) directed against the B-cell form of CD45R and FITC-labeled anti-ICAM-1 (3E2).

Neutrophil Migration Is Impaired in Mutant Mice. To assess the role of ICAM-1 in transendothelial migration, a peritonitis study was initiated. The total number of neutrophils in the peritoneal cavity and the percentage of neutrophils relative to all leukocytes in the exudate were reduced in mutant animals, whereas the neutrophil count in the blood 3 hr after thioglycollate injection was even more elevated than in the resting state (Fig. 4). Analysis of peritonitis at 8 hr revealed 76% neutrophils in +/+ animals ($n = 4$) and 43% neutrophils in −/− animals ($n = 6$), indicating the alteration is not simply a delay in emigration.

Contact Hypersensitivity Is Reduced in Mutant Mice. Since ICAM-1 is thought to be important in lymphocyte interac-

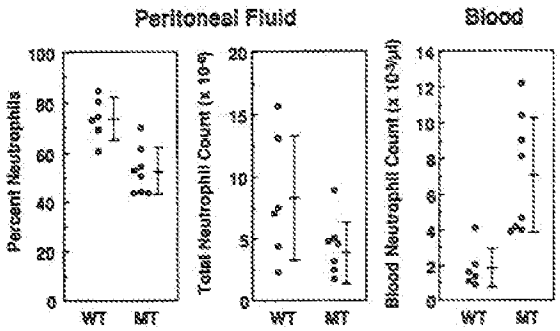

FIG. 4. Altered response to chemical peritonitis in mutant mice. Wild-type (+/+) or homozygous mutant (−/−) mice were injected i.p. with 1 ml of fluid thioglycollate medium and sacrificed after 3 hr. The mean ± SD are shown as bars and from left to right are as follows: 73.8 ± 8.4 for +/+ and 52.6 ± 9.5 for −/− for the percentage of neutrophils in peritoneal fluid ($P = 0.001$), $8.3 \pm 5.0 \times 10^6$ for +/+ and $3.9 \pm 2.5 \times 10^6$ for −/− for the total neutrophils in peritoneal fluid ($P = 0.09$), and $1.85 \pm 1.1 \times 10^3$ per μl for +/+ and $7.07 \pm 3.2 \times 10^3$ per μl for blood neutrophil count ($P = 0.002$). Analysis of peritoneal fluid from four +/+ and four −/− animals without instillation of thioglycolate revealed $0.6–3.0 \times 10^6$ cells per animal with a mean of 4.2% neutrophils for +/+ animals and 1.1% neutrophils for −/− animals.

tions, we examined the ability of the ICAM-1-deficient animals to generate a contact hypersensitivity response. Mice were challenged at 7–19 weeks of age with application of DNFB to one ear 5 days after sensitization by two applications of DNFB to the abdomen. Naive animals received the challenge to the ear but do not undergo abdominal sensitization with DNFB. Maximal ear swelling in all test groups occurred 24 hr after challenge as reported (33) and was reduced by 74% in homozygous mutant animals as shown in Table 1 ($P < 0.0001$, unpaired T test). Histologic study of punch biopsies of the ears confirmed the difference in thickness, and sections from sensitized wild-type animals revealed prominent edema separating normal tissue structures and a moderately dense infiltrate of lymphoid cells and neutrophils (not shown). Both of these changes were essentially absent in mutant animals. These studies indicate that ICAM-1 plays a prominent role in mediation of contact hypersensitivity and demonstrate a significant inflammatory abnormality in the mutant animals.

ICAM-1-Deficient Cells Are Defective as Stimulators in the MLR. In the MLR, the activating stimulus is the foreign histocompatibility antigen expressed on allogeneic stimulator cells, and a proliferative T-cell response is induced. Previous studies demonstrated that antibodies to ICAM-1 inhibit the MLR but did not distinguish the role of its expression on stimulator cells in comparison with the role of induced ICAM-1 expression on responder T cells. Unfractionated spleen cells were irradiated and used as stimulators while T lymphocytes were isolated from spleen for use as responder cells. Cells from wild-type and homozygous mutant mice were of hybrid (C57BL/6J × 129/Sv) background (both $H\text{-}2^b$). Allogeneic cells expressing $H\text{-}2^d$ were isolated from BALB/c mice. The normal and homozygous mutant T lymphocytes responded equally well to allogeneic stimulation with irradiated BALB/c cells and a wide range of concentration of responder cells; for example, mean incorporation was 79,900 cpm for wild-type cells and 68,100 cpm for mutant cells with 5×10^6 responder cells and 4×10^6 BALB/c stimulator cells. However, cells isolated from the spleen of homozygous mutant animals demonstrated a marked reduction in the ability to function as stimulator cells with a wide range of concentrations of BALB/c responder cells (Fig. 5). These data show that the defect involves the function of ICAM-1 primarily or exclusively on the stimulator cells as opposed to the responder cells.

DISCUSSION

The *Icam-1* gene was disrupted by homologous recombination, and homozygous mutant mice are viable but show an absence of surface expression of ICAM-1. The phenotype in the mice might be expected to resemble that seen in CD18 deficiency in humans and animals (34, 35), but the phenotype might be milder, since β_2 integrins are the only proven counterreceptors for ICAM-1, although ICAM-2 and ICAM-3 are suggested to serve as counterreceptors for LFA-1 (36–38), and Mac-1 is the receptor for the iC3b component of complement. The ICAM-1-deficient mice display some phenotypic features similar to partial deficiency of CD18 in human and mice (29), including a mild increase in neutrophil count and impaired neutrophil emigration.

Table 1. Impaired contact hypersensitivity in ICAM-1-deficient mice

Genotype	$\Delta T^* \times 10^2$, mm ± SD	
	Naive	Sensitized
Wild type	0.9 ± 1.5 (n=18)	16.1 ± 7.5 (n=15)
Homozygous mutant	1.0 ± 2.3 (n=14)	5.8 ± 4.7 (n=26)

*ΔT = (ear thickness 24 hr after elicitation) − (ear thickness before DNFB challenge). The difference between sensitized mutant and wild-type ΔT is significant; $P < 0.0001$, unpaired t test; 74% reduction.

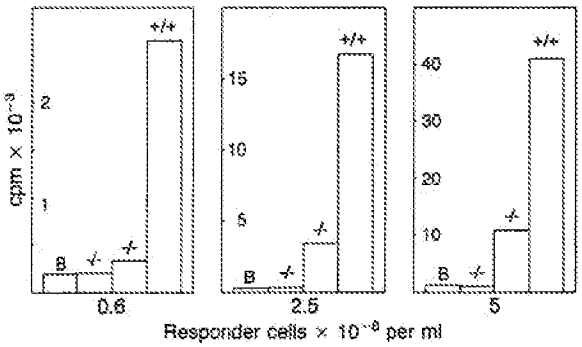

FIG. 5. Stimulator cell capacity of mutant cells in MLR. Spleen cells were isolated from wild type (+/+) or homozygous mutant (−/−) hybrid (129/Sv × C57BL/6J) mice and from BALB/c (B) mice and were irradiated for use as stimulator cells. Responder cells were from BALB/c mice.

In the chemical peritonitis studies of ICAM-1-deficient mice, the reduction in neutrophils in the peritoneal exudate and the accumulation of granulocytes in the blood is indicative of decreased transendothelial migration of neutrophils in the mutant mice. Since the migration defect in the mutant mice is not complete, there is evidence for an ICAM-1-independent mechanism for transendothelial migration. The results are consistent with *in vitro* studies in which mAb to ICAM-1 inhibited transendothelial migration by only 55%, while mAb to CD18 inhibited by 90% (39).

The ICAM-1-deficient animals exhibited a 74% suppression of contact hypersensitivity. Based on the MLR results with ICAM-1-deficient cells, it seems probable that the defect in contact hypersensitivity will involve the afferent or sensitization phase of the response. The contact hypersensitivity data are compatible with the hypothesis that ICAM-1 is a critical accessory molecule for T-cell function. It is also possible that the defect in the contact hypersensitivity response may be caused by abnormalities of migration involving the antigen-presenting cell or the T cell.

The data from the MLR suggest that the deficiency of ICAM-1 on the stimulator cells and not the responder cells is responsible for the diminished response. The critical step for T-cell activation is the recognition of antigen peptides in association with MHC molecules by the T-cell receptor (TCR-CD3). However, cell adhesion molecules are thought to play an important role in providing costimulatory signals between lymphocytes or in enhancing lymphocyte interactions (40). Although the interaction of CD2 and CD58 (LFA-3) is thought to be an important costimulatory event for generation of an immune response, mice with a disrupted *CD2* gene demonstrated normal immune responses (41). It was suggested that the ICAM-1/LFA-1 or other interactions might provide a redundant adhesive function in the CD2-deficient mice. The severe defect (up to 100%) in the ability of ICAM-1-deficient T cells to function as stimulator cells in the MLR is consistent with an important costimulatory role for ICAM-1. The expression of ICAM-1 in antigen presentation can be a decisive factor in determining whether a T-cell response will occur. This interpretation is supported by transfection studies expressing ICAM-1 and HLA-DR in L cells (42). L cells expressing HLA-DR alone failed to activate T cells, while cells expressing HLA-DR and ICAM-1 were effective. Transfection with ICAM-1 was also effective in correcting the defect in mutagenized clones of antigen presenting cells (43).

The ICAM-1-deficient mice and other mice with gene-targeted mutations in cell adhesion molecules should be valuable resources for the study of inflammatory responses *in vivo*. There is considerable interest in the hypothesis that decreased expression or function of cell adhesion molecules might result in reduced susceptibility to common, multifactorial diseases that have inflammation as a component including arthritis, diabetes mellitus, inflammatory bowel disease, asthma, atherosclerosis, and various other autoimmune and inflammatory diseases. Monoclonal antibodies that block function of ICAM-1 have been shown to reduce inflammatory or immune responses in a variety of disease models (44–47), but these antibodies may induce biological responses apart from their role in blocking adhesion. The mutant mice offer an important alternative strategy to assess the role of ICAM-1, and the mice are more suitable for study of chronic inflammatory disease processes.

We thank Don Anderson and Robert Rothlein for helpful discussions; Eric Sandberg, Paul Stein, and Hon-Man Lee for help and advice with immunological studies; Wendy Schober and Betsy Priest for technical assistance; and Grace Watson for preparation of the manuscript. These investigations were supported by grants from the National Institutes of Health (AI 32177 to A.L.B., HL 02537 to C.M.B., AI 32609 to S.S.R., and HL 42550 to C.W.S.).

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Appendix B

Role of ICAM-1 and ICAM-2 and alternate CD11/CD18 ligands in neutrophil transendothelial migration

Andrew C. Issekutz,* Derek Rowter,* and Timothy A. Springer†

*Departments of Pediatrics, Pathology and Microbiology/Immunology, Dalhousie University, Halifax, Nova Scotia, Canada; and †Center for Blood Research and Department of Pathology, Harvard Medical School, Boston, Massachusetts

Abstract: **We evaluated the relative contribution of ICAM-1 and ICAM-2, known ligands on endothelium for LFA-1 and Mac-1, in spontaneous neutrophil (PMN) transendothelial migration (TEM) across IL-1-activated HUVEC monolayers or TEM induced by C5a or IL-8 across unstimulated HUVEC grown on polycarbonate filters. Adhesion blocking mAb to ICAM-1 [R6.5 F(ab)$_2$] or ICAM-2 [CBR IC2/2 F(ab)$_2$] tended to inhibit TEM under each condition but, in general, inhibition was significant only with both ICAM-1 and ICAM-2 blockade. mAb to LFA-1 partially inhibited migration to C5a or IL-8 across unstimulated HUVEC and inhibition was not altered by additional treatment of HUVEC with mAbs to ICAM-1 and -2. In contrast, with IL-1 HUVEC, mAb to ICAM-1 significantly inhibited this LFA-1-independent TEM. mAb to Mac-1 alone partially inhibited TEM and, when combined with mAb to LFA-1, migration was almost completely blocked with all TEM conditions tested. The contribution of alternate ligands for Mac-1 in mediating Mac-1-dependent but ICAM-1/-2-independent C5a-induced TEM was examined using anti-LFA-1-treated PMN and anti-ICAM-treated resting HUVEC. Addition of RGD peptides, fibronectin, fibrinogen, heparins, collagens alone or in combination, even to heparinase-treated HUVEC, did not inhibit this Mac-1-mediated PMN TEM. The results indicate that: (1) LFA-1 mediates PMN TEM primarily by interaction with ICAM-1 and ICAM-2; (2) ICAM-2 may function in concert with ICAM-1 in this role, especially on unstimulated endothelium, and (3) Mac-1 on PMN also plays a major role in TEM and can utilize yet to be identified ligands distinct from ICAM-1 or -2, especially on unstimulated endothelium.** *J. Leukoc. Biol.* **65: 117–126; 1999.**

Key Words: *adhesion molecule · leukocyte · endothelium · Mac-1 · CD102 · LFA-1*

INTRODUCTION

A characteristic feature of acute inflammation is the migration of leukocytes, especially polymorphonuclear leukocytes (PMN), from blood into the involved tissues. In part, this migration is believed to be induced by chemotactic factors produced in the inflamed tissue, which bind to specific receptors on PMN and activate intracellular signal transduction leading to adhesion of the leukocyte to vascular endothelium and a motile response [1–4].

It is now also recognized that leukocyte migration has an important endothelial cell-dependent component. Activation of endothelial cells by cytokines, such as interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and bacterial products such as endotoxin (lipopolysaccharide, LPS) leads to increased PMN adhesion to the endothelium and transendothelial migration [5–7]. Adhesion molecules, such as P-selectin, E-selectin, and intercellular adhesion molecule-1 (ICAM-1) are in part responsible for this leukocyte endothelial cell interaction [5–7]. These molecules are expressed on the endothelium and interact with sialyl Lewis^x carbohydrate-containing molecules, and the CD11/CD18 integrins (β_2 integrins) on the PMN in this process.

The transendothelial migration of PMN *in vitro*, via the chemotactic factor-dependent or the endothelial cell-dependent mechanisms, appears to have a nearly absolute requirement for the presence and function of the CD11/CD18 leukocyte adhesion molecule complex [6, 8–10]. Furthermore, *in vivo* assessment of leukocyte migration to sites of infection or inflammation, especially in the skin, have demonstrated failure of leukocyte mobilization in patients with congenital CD18 deficiency or in experimental animals treated with anti-CD18 mAbs [11–14]. Of the β_2 or CD18 complex, CD11a/CD18 and CD11b/CD18, also known as LFA-1 and Mac-1, respectively, each appear to be important and in combination mediate all CD11/CD18-dependent transendothelial migration *in vitro* [5–7] and PMN accumulation in at least some types of inflammatory reactions *in vivo* [12, 15, 16].

The LFA-1 and Mac-1 β_2 integrins are known to bind to ICAM-1 and LFA-1 and, according to one report, Mac-1 also may bind to ICAM-2 [17–21]. Both of these ICAMs are

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Abbreviations: PMN, polymorphonuclear neutrophils; TEM, transendothelial migration; HUVEC, human umbilical vein endothelial cells; IL-1, interleukin-1; TNF-α, tumor necrosis factor α; LPS, lipopolysaccharide; HSA, human serum albumin; PBS, phosphate-buffered saline; FCS, fetal calf serum.

Correspondence: Dr. Andrew C. Issekutz, Department of Pediatrics, Dalhousie University

Infection Immunology Research Labs., IWK Grace Health Centre, 5850 University Ave., Halifax, NS B3J 3G9. E-mail: aissekutz@iwkgrace.ns.ca

Received May 6, 1998; revised October 1, 1998; accepted October 3, 1998.

constitutively expressed on endothelial cells, including human umbilical vein endothelium (HUVEC). ICAM-1 on HUVEC has been reported to contribute to PMN transendothelial migration [8, 9], but the role of ICAM-2 in this process and its importance in comparison to ICAM-1 has not been determined. Furthermore, although Mac-1 can bind to ICAM-1, it can also recognize numerous other ligands, including plasma proteins, e.g., Factor X, C3bi, fibrinogen, fibronectin, and other Arg-Gly-Asp (RGD) sequence proteins, other extracellular matrix proteins (collagen, laminin), heparin like glycosaminoglycans, carbohydrate structures related to β-glucan, and several microbial products [6, 22–28]. These Mac-1 ligand interactions have been observed primarily with adhesion studies with PMN or with purified proteins. The importance of the Mac-1/ICAM-1 interaction and of interactions with these other ligands in mediating PMN transendothelial migration has not been defined. This study was aimed to address these questions and reports that ICAM-2 is a major contributor with ICAM-1 to LFA-1-mediated PMN transendothelial migration. Furthermore, Mac-1-mediated transendothelial migration induced by chemotactic factors is ICAM-1 independent and only partly ICAM-1 dependent when endothelium is activated with IL-1.

MATERIALS AND METHODS

Monoclonal antibodies

The mAbs used included mAb 60.3 (IgG2a; a gift from Bristol-Myers Squibb, Seattle, WA) [29, 30], mAb LPM19c (IgG1; anti-CD11b, a gift from K. Pulford, Oxford, UK) [31], and mAb TS1/22 (IgG1, anti-CD11a/CD18, or LFA-1) [32], which are all known to block adhesion functions of their respective CD11/CD18 antigens. The mAbs W6/32 (IgG2a anti-HLA-class I framework), 3C10 (IgG1 anti-CD14), 543 (IgG1, anti-CR1), and mAb TS1/22 were obtained from the American Tissue Culture Collection (Bethesda, MD). The mAbs reactive with ICAM-1 were mAb R6.5 [IgG1 as $F(ab)_2$] [13], mAb CBR-IC 1/13, and CBR-IC 1/11, the latter two reacting specifically with the domain-3 binding region for Mac-1 [33]. mAb CBR-IC2/2 [IgG1 as $F(ab)_2$] recognizing ICAM-2 [18] and mAb BBI1 (IgG2b, gift from Dr. R. Lobb, Biogen, Cambridge, MA) against E-selectin [34] were also employed.

Reagents

Recombinant human IL-1α, which had a specific activity of 4×10^7 U/mg, was a gift from Dr. D. Urdal (Immunex Corp., Seattle, WA). Recombinant human IL-8 (NAP-1) were kind gifts from Sandoz Pharmaceutical (Vienna, Austria). Each of the cytokines was diluted immediately before use in 0.1% LPS-free human serum albumin (HSA; Connaught Labs, Don Mills, ON) in phosphate-buffered saline (PBS). Recombinant human C5a was a gift from CIBA-Geigy Pharmaceuticals (Summit, NJ). The following were purchased from Sigma Chemical Co. (St. Louis, MO): Factor X, β-glucan, fibrinogen and its γ-peptide, fibronectin, Type II bovine nasal collagen and Type IV human placental collagen, mouse laminin, and heparinase I and III. The GRGDSP peptide was from Bachem Fine Chemicals (Torrance, CA).

Human PMN purification

Human PMNs were purified as described previously [35, 36] from ACD-heparin-anticoagulated venous blood of healthy donors. Briefly, red cells were sedimented with 6% dextran-saline (Abbott Labs, Montreal, Canada), leukocyte-rich plasma was collected, and leukocytes were labeled with $Na_2{}^{51}CrO_4$ (Amersham, Oakville, Ontario, Canada). PMNs were then purified by discontinuous Percoll gradient centrifugation, washed, and resuspended to 10^6 PMN/mL in RPMI-1640, 0.5% HSA, 10 mM HEPES, pH 7.4. This method yielded PMNs of ≥95% purity with essentially no red cell contamination and ≥98% cell viability.

Endothelial cell cultures

HUVEC were isolated and cultured in flasks as described by Jaffe et al. [37] and grown on filters as previously described by us [35, 36]. Briefly, endothelial cells were isolated from umbilical cords after treatment with 0.5 mg/mL collagenase (Cooper Biomedical, Mississauga, Ontario, Canada) in 0.01 M PBS (pH 7.4) and grown in RMPI-1640 (Sigma) containing 2 mM L-glutamine, 2-mercaptoethanol, sodium pyruvate, and penicillin B/streptomycin and supplemented with 20% fetal calf serum (FCS; Hyclone, Logan, UT), 25 μg/mL endothelial cell growth supplement (Collaborative Research, Lexington, MA), and heparin (45 μg/mL; Sigma). This is referred to as growth medium. Cells were cultured in gelatin-coated culture flasks (NUNC, GIBCO). The HUVEC were detached using 0.025% trypsin, 0.01% EDTA (Sigma) and cultured on PVP-free polycarbonate filters bearing 5-μm pores in Transwell culture plate inserts (6.5-mm diameter, Transwell 3421; Costar, Cambridge, MA). The filters were first prepared by coating with 0.01% gelatin (37°C, 18 h) followed by application of 3 μg of human fibronectin (Collaborative Research) in 50 μL of water at 37°C for 2 h. Fibronectin was then replaced by HUVEC (1.5×10^5 cells), from the first or second passage, added above the filter in 0.1 mL of growth medium and 0.6 mL of growth medium was added to the lower compartment beneath the filter. The HUVEC formed a tight permeability barrier in 5–6 days and were evaluated for confluence before use by ^{125}I-labeled HSA diffusion as previously described [35].

PMN transendothelial migration

Migration assays were performed as described previously [35, 36]. Briefly, HUVEC monolayers on the filters and the lower compartments were washed with RPMI 1640 and they were transferred to a new, clean well (lower compartment). To this well, 0.6 mL of RPMI-1640, 10 mM HEPES, 0.5% HSA was added containing the chemotactic stimulus (C5a, IL-8). Before immersion of the HUVEC-filter unit, 0.1 mL of medium containing 1×10^5 labeled PMNs was added above the HUVEC. After incubation (75 min at 37°C, 5% CO_2) migration was stopped by washing the upper compartment twice with 0.3 mL of RPMI-1640 to remove nonadherent PMNs. The undersurface of the filter was wiped with a cotton swab saturated with ice-cold PBS-0.2% EDTA solution and this was added to the lower compartment. The cells that spontaneously detached from the undersurface of the filter or were removed by the swab were lysed by addition of 0.5% Triton X-100 and all the ^{51}Cr released in the lower compartment and on the swab was quantitated. The results are expressed as the percentage of the total ^{51}Cr PMNs added above the HUVEC that migrated through the HUVEC-filter unit. All the stimulation conditions were performed with triplicate replicates.

Antibody treatments

In some experiments, ^{51}Cr PMNs were treated for 20 min at room temperature with the mAbs indicated at saturating concentrations (20–40 μg/mL), as determined by immunofluorescence flow cytometry, and then tested for migration in the presence of the antibody. None of the mAb treatments caused PMN aggregation or activation as assessed by oxidative burst using luminol dependent chemiluminescence or bipolar shape change. Other reagents were added to the PMN suspension just before adding them to the HUVEC. In some experiments, the HUVEC were treated for 40 min at 37°C with saturating concentrations of mAbs as determined by enzyme-linked immunoabsorbent assay followed by the addition of the ^{51}Cr-labeled PMNs. These mAbs were present throughout the migration period as well. In some cases, the HUVEC was treated with heparinase III (1 U/mL) or I (1 U/mL) as reported previously [26] for 40 min before performing the PMN migration assay.

Statistical analysis

Data were analyzed by analysis of variance followed by the Tukey test of multiple comparisons. P values exceeding 0.05 were not considered significant.

RESULTS

Effect of ICAM-1 and ICAM-2 blockade on chemotactic factor-induced PMN transendothelial migration

The contribution of the CD11/CD18 integrins, LFA-1 and Mac-1 on PMN and of ICAM-1 and ICAM-2 on endothelium to PMN transendothelial migration induced by the potent chemotactic factor, C5a, was investigated as shown in **Figure 1**. C5a at an optimal chemotactic concentration of 2×10^{-9} M, predetermined in previous studies [36], induced 67% of PMN to transmigrate across the HUVEC and filter barriers. Blocking LFA-1 adhesion function with the mAb TS1/22 significantly but only partially inhibited this response. Similarly, blocking Mac-1 with mAb LPM19C inhibited transmigration to a comparable degree. The combination of these two mAbs essentially eliminated any PMN transendothelial migration in response to C5a, as also reported previously using this or similar transendothelial migration systems [9, 10, 36]. To investigate the role of ICAM-1 and ICAM-2 on HUVEC in PMN transmigration, the adhesion function blocking mAbs R6.5 and CBR-IC2/2, respectively, were employed in their $F(ab)_2$ forms. As shown in Figure 1, anti-ICAM-1 or anti-ICAM-2 treatment of the HUVEC had a slight but not significant inhibitory effect on transmigration when the PMN were not mAb treated. Addition of mAb to E-selectin to the anti-ICAM-1 and -2 mAbs had no effect on migration (not shown). However, combination of anti-ICAM-1 and anti-ICAM-2 mAbs had a significant inhibitory effect, but still only decreased the migration from 67 to 48% of PMN transmigrating. Treatment of PMN with anti-LFA-1 mAb did not further inhibit migration when the HUVEC were treated with anti-ICAM-1 + ICAM-2 mAbs. In contrast, treatment of the PMNs with anti-Mac-1 mAb abolished PMN transendothelial migration when ICAM-1 and -2 on the HUVEC were blocked. These findings indicate that the ICAM-1 + ICAM-2 blocking mAbs effectively blocked the LFA-1 pathway and that in the presence of anti-ICAM-1 + anti-ICAM-2 ± anti-LFA-1 blockade, all of the PMN transendothelial migration was mediated by Mac-1.

Figure 2 shows the results with use of the IL-8 chemotactic factor to induce PMN transendothelial migration. IL-8 induced optimal PMN transmigration at a concentration of 5×10^{-9} M), but this response was significantly weaker than with C5a, inducing about 42% of PMN to transmigrate. Anti-LFA-1 treatment of the PMN inhibited this response by approximately 50%. Antibody to Mac-1 inhibited PMN migration to a somewhat greater degree, i.e. by approximately 75%, and the combination of anti-LFA-1 with anti-Mac-1 mAbs decreased migration to virtually the unstimulated level, i.e. to about 4%. Treatment of the HUVEC with anti-ICAM-1 mAb [R6.5 $F(ab)_2$] or anti-ICAM-2 mAb alone tended to inhibit migration, but this was not significant. In the IL-8-induced transmigration, addition of anti-ICAM-1 mAb to anti-ICAM-2 mAb had a statistically significant additive inhibitory effect, blocking migration by more than 50% and to a level comparable with anti-LFA-1 treatment of the PMN. Furthermore, LFA-1 mAb treatment of PMNs in combination with anti-ICAM-1 + ICAM-2 treatment of HUVEC had no further inhibitory effect compared to either anti-ICAM-1 + -2 treatment or anti-LFA-1 treatment alone. In marked contrast, treating the PMNs with antibody to Mac-1 completely blocked their migration when ICAM-1 and ICAM-2 on the HUVEC was also blocked. These observations suggest

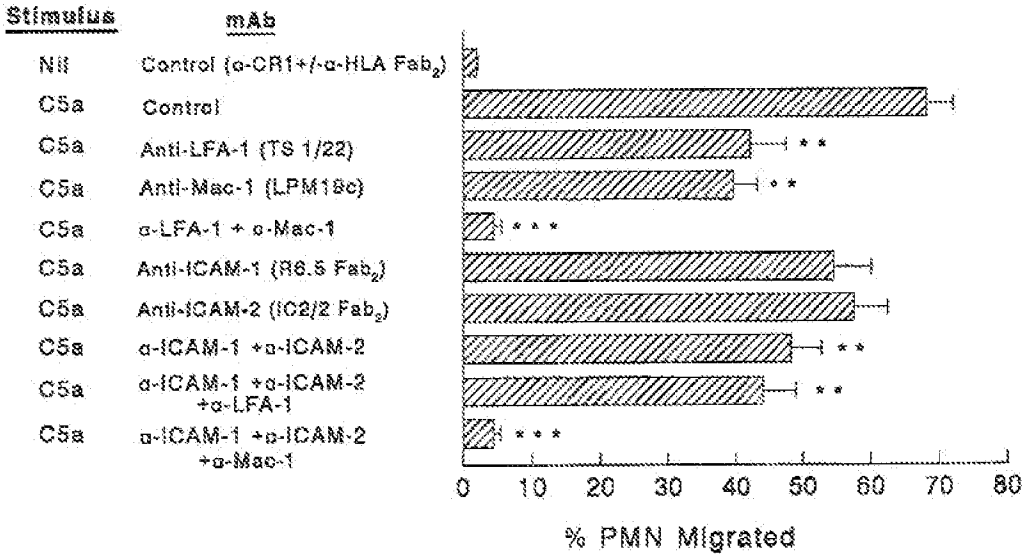

Fig. 1. The effect of antibody to LFA-1, Mac-1, ICAM-1, and ICAM-2 on PMN transendothelial migration. The migration of ^{51}Cr-labeled PMN across unstimulated HUVEC monolayers was quantitated as described in Materials and Methods. Migration was induced by C5a (2×10^{-9} M) added to the lower compartment beneath resting HUVEC monolayers. PMN were either treated with mAb to LFA-1 (TS1/22) or to Mac-1 (LPM19c) or in combination for 20 min (22°C) before addition above the HUVEC monolayers. Control mAb was an anti-CR-1 mAb (543). Where indicated, the HUVEC were pretreated with anti-ICAM-1 mAb [R6.5 $F(ab)_2$] or anti-ICAM-2 [CBR-IC2/2 $F(ab)_2$, 30 μg/mL] for 30 min before addition of PMN. Control mAb for the HUVEC treatments was an anti-HLA Class I [mAb W6/32 $F(ab)_2$]. PMN migration time was 75 min. Values are mean ± SEM of five to eight experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control mAb treatments of the PMN and/or HUVEC.

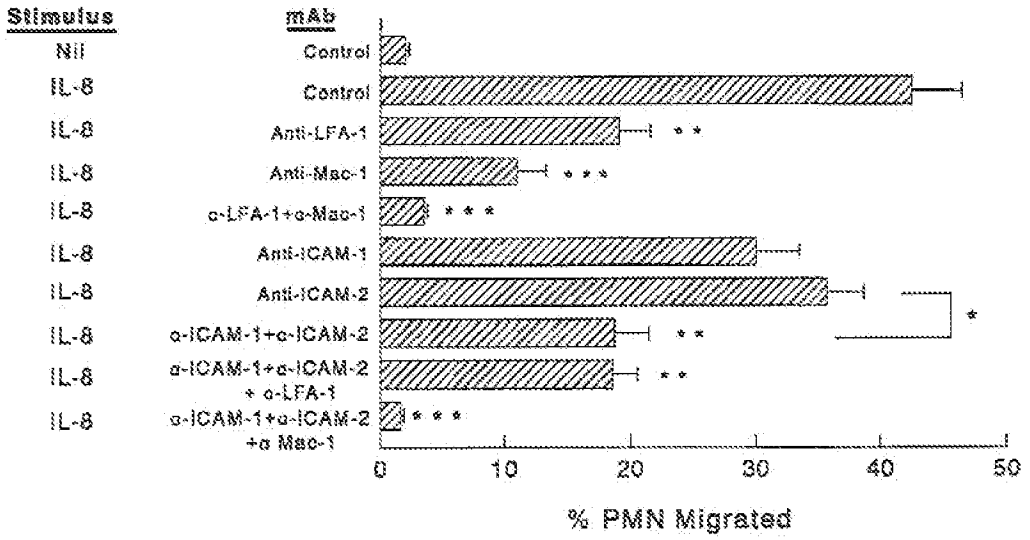

Fig. 2. Effect of antibody to LFA-1, Mac-1, ICAM-1, and ICAM-2 on PMN transendothelial migration induced by IL-8. Migration of PMN across unstimulated HUVEC monolayers was induced by IL-8 (5×10^{-8} M) added to the lower compartment beneath the HUVEC monolayer. Migration time and mAb treatments were as in Figure 1 using the same mAbs. Values are mean ± SEM of four to six experiments. P values are as in Figure 1.

that different mechanisms for transendothelial migration function in concert and these involve an LFA-1-mediated pathway on the PMN engaging an ICAM-1/ICAM-2 pathway on the endothelium and a Mac-1 pathway on the PMN, which may utilize ligands other than ICAM-1 and -2.

Investigation of Mac-1 ligands contributing to the Mac-1-mediated PMN transendothelial migration

The results in Figures 1 and 2, using C5a and IL-8, indicate that Mac-1 on PMN can mediate 50–60% of the transendothelial migration. Therefore, we investigated, using C5a as the chemoattractant, the potential involvement of some of the putative ligands for Mac-1. Mac-1 has been reported to bind to ICAM-2 [17], to domain-3 of ICAM-1, which is distinct from the domain-1 binding region for LFA-1 [6, 19, 20], to RGD peptides and similar sequences in fibrinogen and fibronectin, as well as other extracellular matrix proteins such as collagens, laminin, as well as to glycosaminoglycans related to heparan sulfates [22–28]. Also Mac-1 has a lectin binding region that recognizes carbohydrate structures such as β-glucan [38]. To determine which of these interactions alone or in combination may be mediating PMN transendothelial migration via the Mac-1 pathway, PMN that were treated with mAb to LFA-1 were added to HUVEC monolayers, which were untreated, treated with anti-ICAM-1 mAb R6.5 $F(ab)_2$, or with other antibodies to HUVEC adhesion molecules, as shown in **Figure 3**. As expected, anti-LFA-1-treated PMN were partially inhibited in their transmigration across the HUVEC in response to C5a. Treating the endothelium with anti-ICAM-1 mAb R6.5 $F(ab)_2$, which is known to block the interaction of LFA-1 with ICAM-1 and also the interaction of Mac-1 with ICAM-1 [13, 25], did not significantly inhibit further compared with anti-LFA-1 alone. Adding treatment with mAb CBR-IC2/2 of the HUVEC, which is reported to at least partially inhibit Mac-1 binding to ICAM-2 [17], did not inhibit transmigration.

Because Mac-1 recognizes a different domain on ICAM-1 from LFA-1, the possibility existed that the R6.5 mAb may be more effective at blocking the LFA-1/ICAM-1 interaction than the Mac-1/ICAM-1 interaction at domain 3 of ICAM-1. Therefore, two other mAbs to ICAM-1 (clones CBR-IC1/13 and CBR-IC1/11), which are known to recognize epitopes in domain 3 of ICAM-1 and block Mac-1 binding [33], were also used. However, these antibodies alone (not shown) or in combination with the R6.5 mAb, had no additional inhibitory effect on the Mac-1-mediated transmigration, as shown in Figure 3.

Having observed no requirement for ICAM-1 or -2 in the Mac-1-mediated PMN migration, the role of other putative ligands was investigated. In these experiments, the addition of the RGD peptide GRGDNP to the PMN suspension before and during the PMN transmigration assay, at concentrations (0.1–1 mM) known to inhibit Mac-1 binding to RGD peptides [24, 28, 39] had no effect on migration. Fibrinogen is readily bound by activated Mac-1, but the presence of free fibrinogen or the fibrinogen gamma peptide, which contains a Mac-1 recognition region [40], with or without blockade of ICAM-2 in the presence of anti-ICAM-1, did not inhibit Mac-1-mediated transmigration either. Similarly, a range of concentrations of two different forms of heparin, which are reported to inhibit Mac-1-heparin and glycosaminoglycan binding [22, 26], did not inhibit Mac-1-mediated PMN transmigration. Inclusion of type 2 and type 4 collagens (or laminin, not shown), which are known to be ligands for Mac-1-mediated PMN adhesion [24, 28], had no effect on the PMN migration response. The lectin binding domain of Mac-1 has been reported to be involved in phagocytic recognition of β-glucan on yeast particles and may also be involved in intra-membrane molecular associations between Mac-1 and some GPI linked proteins [38, 41–43]. To evaluate the contribution of this interaction, soluble β-glucan was included in the PMN suspension during the migration. However, β-glucan did not inhibit PMN transendothelial migration via the Mac-1 pathway.

Recent studies by Diamond et al. [22] and Coombs et al. [26]

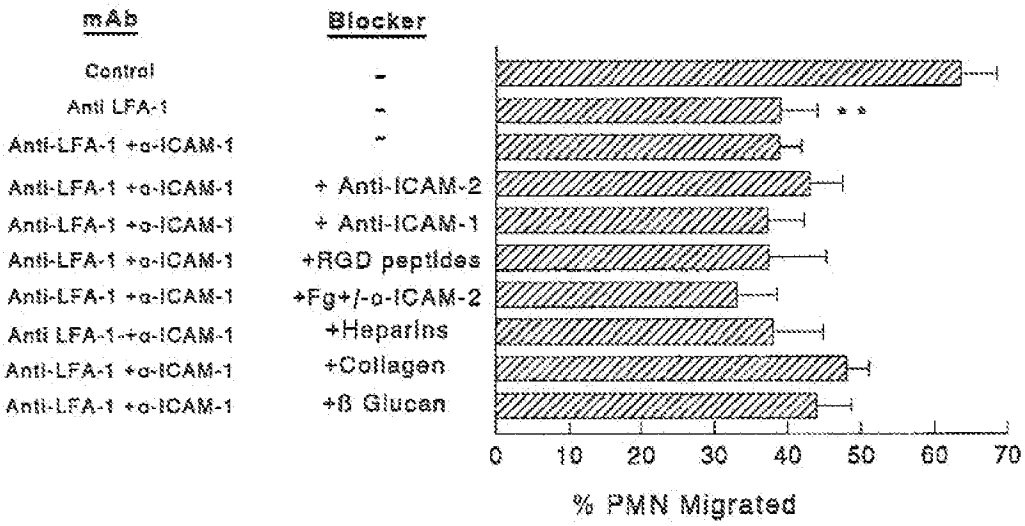

Fig. 3. Effect of blocking putative Mac-1 ligand interactions on PMN transendothelial migration induced by C5a. PMN migration was induced as in Figure 1 using C5a. PMN were treated with control mAb or with anti-LFA-1 (TS1/22) and the HUVEC were treated with anti-ICAM-1 mAb clone R6.5 $F(ab)_2$ as indicated in the column at left, as in Figure 1. The column labeled blocker shows additional treatments of either the HUVEC using anti-ICAM-2 mAb (CBR-IC2/2 $F(ab)_2$) or additional anti-ICAM-1 mAbs reactive with domain-3 (CBR-IC1/13 or IC1/11). Other blocking treatments included GRGDSP peptide (0.1–1 mM) human fibrinogen (Fg), or Fgγ peptide (100–500 μg/mL, results pooled), heparins (porcine intestinal mucosa, bovine lung, or low molecular weight heparin at 10–1000 μg/mL, results pooled), or bovine nasal (Type II) or human placental (Type IV) collagen (50–500 μg/mL), or β-glucan (50–200 μg/mL) added to the PMN suspension 20 *min* before addition to the HUVEC. All mAbs and agents were present throughout the migration assay. Values are mean ± SEM of four to eight experiments. $^{**}P < 0.01$.

have shown that heparin and heparan sulfate glycosaminoglycans may be important ligands for Mac-1. Glycosaminoglycans are abundant on vascular endothelium. Therefore, we investigated the potential role of these structures in Mac-1-mediated transendothelial migration. For these experiments, heparinase III treatment of the HUVEC was employed, using conditions reported previously to block Mac-1-glycosaminoglycan-mediated adhesion to stromal cells *in vitro* [26]. Heparinase III treatment of the HUVEC had no deleterious effect on the permeability of the monolayer and did not alter baseline or C5a-induced PMN transmigration (not shown). However, when the system was designed to quantitate PMN transmigration via the Mac-1 pathway, i.e. by using anti-LFA-1-treated PMN and anti-ICAM-1 [R6.5 $F(ab)_2$] -treated HUVEC, heparinase III treatment of the HUVEC had no effect on transmigration. Furthermore, adding fibrinogen γ peptide alone or in combination with anti-ICAM-2 mAb or with fibronectin ± β-glucan did not significantly inhibit transmigration. In two experiments, inclusion of Factor X in the blocking treatment of the PMN also did not inhibit PMN transmigration via the Mac-1 pathway (**Figure 4**). In all of these experiments, the permeability of the endothelial monolayer remained comparable to monolayers in which no blocking treatments were used, i.e. there was no adverse effect on the monolayer integrity before and during the assay (not shown).

Finally, we considered that some combination of plasma constituents such as fibrinogen, fibronectin, Factor X, and haptoglobulin, all of which are reported to be ligands for Mac-1 [6, 7, 44, 45], might interact with and regulate Mac-1 recognition of ligands on endothelium by binding to Mac-1 from the soluble phase. To assess this, IL-8 was used as the chemotactic factor, since plasma rapidly inactivated the C5a chemotactic agent as expected. Inclusion of up to 40% plasma in the upper and lower compartment of the chemotactic chamber had no effect on IL-8-induced transendothelial migration (not shown). Furthermore, treating PMNs with antibody to LFA-1 and the HUVEC with anti-ICAM-1 partially inhibited the transmigration, as shown in Figure 2, and in the presence of 40% plasma there was no further inhibition (no plasma control = 18 ± 2.5%; with plasma = 14.2 ± 2.5% of PMN transmigrated; $n = 3$). In the case of both IL-8 and C5a, the degree of LFA-1 versus Mac-1-mediated transmigration was not dependent on the kinetics of migration because terminating the incubations at earlier time points to achieve 50% maximal response, i.e., at 40-min incubation, did not alter the degree of inhibition by anti-LFA-1 or anti-Mac-1 mAb treatment of PMNs. Furthermore, the contribution of ICAM-1 versus ICAM-2 to the migration response was also not affected (not shown).

Contribution of ICAM-1 and ICAM-2 to PMN migration across IL-1-activated HUVEC

Stimulation of HUVEC with IL-1 or TNF-α is known to result in PMN transendothelial migration. As shown in **Figure 5**, activation of HUVEC with IL-1α for 4 h increased PMN transmigration from a background level of 2.5% up to 28%. To investigate the role of ICAM-1 and ICAM-2 on HUVEC, the HUVEC was pretreated with mAb R6.5 or CBR-IC2/2, respectively, as in the previous experiments. Control mAb [anti-HLA-class I, W6.32 $F(ab)_2$] had no effect on migration, as shown in Figure 5. Anti-ICAM-1 mAb significantly inhibited PMN transendothelial migration. Although anti-ICAM-2 mAb did not have a significant effect, the addition of mAb to ICAM-1 had a significant additive inhibitory effect, decreasing the PMN migration from 28 to 11%. Blocking LFA-1 on PMNs with mAb TS1/22 also inhibited PMN transmigration, but not quite as

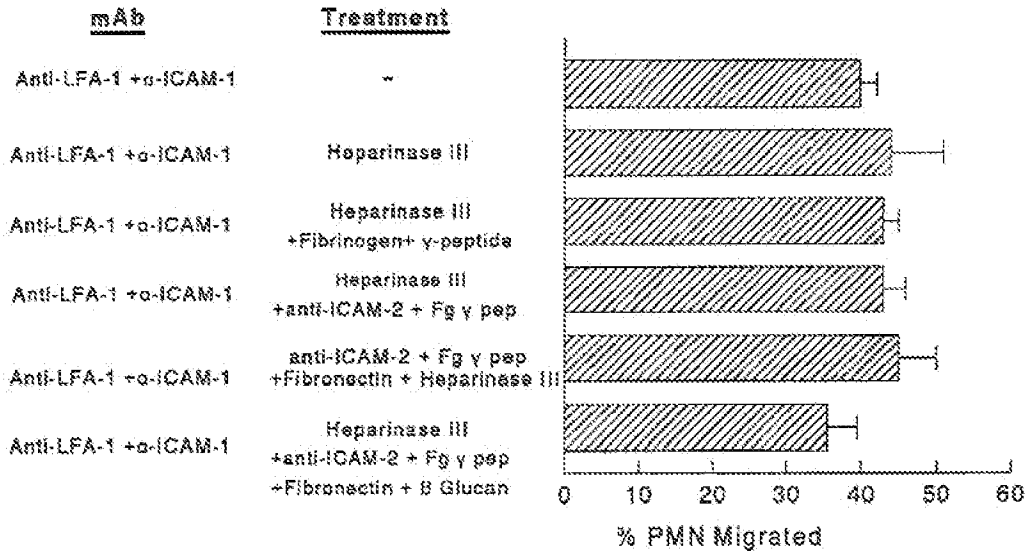

Fig. 4. Effect of heparinase treatment of endothelium on PMN transendothelial migration. The PMN were treated as in Figure 3 with anti-LFA-1 mAb (TS1/22) and HUVEC was treated with anti-ICAM-1 mAb [R6.5 F(ab)$_2$]. Other treatments were as in Figure 2, except that the HUVEC was pretreated with heparinase I and/or heparinase III at 1 U/mL (45 min at 37°C) before addition of antibody-treated PMN. C5a was added to the lower compartment to initiate the migration. Fibrinogen γ peptide and fibronectin were applied to the PMN suspension at concentrations of 200 and 300 μg/mL, respectively. Values are mean ± SEM of three to four experiments.

effectively as blocking ICAM-1 and ICAM-2 on the HUVEC. These results were in contrast to the observations with C5a- or IL-8-induced PMN migration (Figs. 1 and 2) across unstimulated HUVEC. Furthermore, the migration of anti-LFA-1-treated PMN across IL-1-stimulated HUVEC was inhibited significantly further by anti-ICAM-1 mAb [R6.5 F(ab)$_2$] treatment of the HUVEC, suggesting that on IL-1-activated HUVEC, the R6.5 F(ab)$_2$ mAb was blocking an LFA-1-independent migration pathway. This too was in contrast to IL-8 or C5a-induced migration through unstimulated HUVEC where this effect was not observed (Figs. 1 and 2). As shown in Figure 5, antibody to Mac-1 partially inhibited transendothelial migration by about 50% and the combination of anti-LFA-1 and anti-Mac-1 mAbs completely inhibited IL-1-stimulated PMN migration, indicating that this was an LFA-1/Mac-1-mediated migration response as expected from previous reports [8, 9]. It

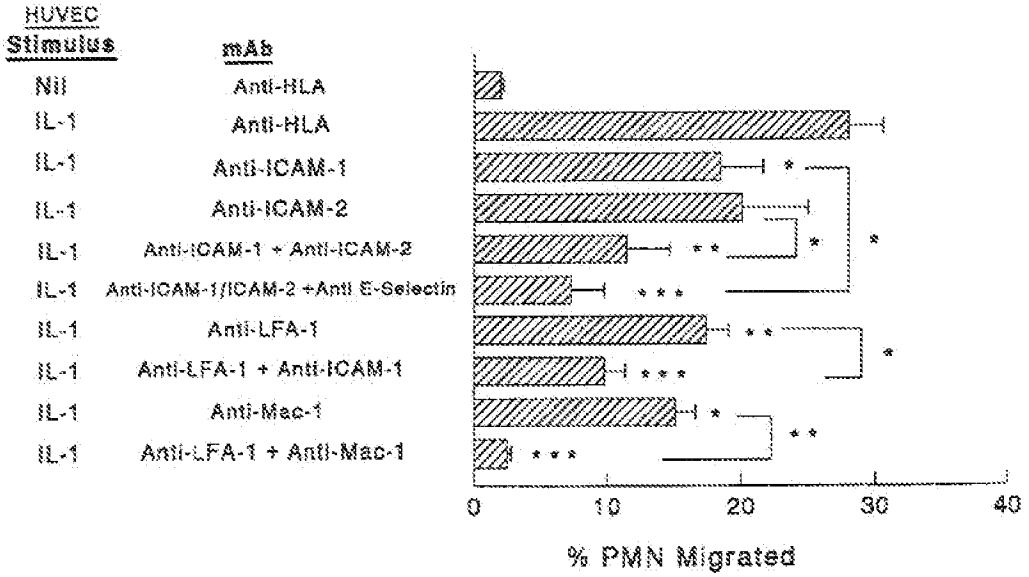

Fig. 5. Effect of antibody to LFA-1, Mac-1, and ICAM-1 and -2 on PMN migration across IL-1-activated endothelium. The HUVEC were stimulated with IL-1α (0.5 ng/mL for 4 h) followed by washing and addition of PMN treated with anti-LFA-1 mAb (TS1/22) or anti-Mac-1 (LPM19c) as in Figure 1. The HUVEC were pretreated for 30 min with anti-ICAM-1 [R6.5 F(ab)$_2$], anti-ICAM-2 (CBR-IC2/2), anti-E-selectin (BB11, 20 μg/mL) mAb alone or in combination as indicated. Control mAb was anti-HLA Class I mAb [W6/32 F(ab)$_2$]. PMN migration was quantitated after 75 min. Values are mean ± SEM of five to six experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

should be indicated that neither anti-LFA-1 + anti-ICAM-1 nor anti-ICAM-1 + anti-ICAM-2 treatments decreased absolute PMN migration below 10%, i.e. approximately one-third of the response remained. Adding anti-E-selectin mAb to treat the HUVEC along with the anti-ICAM-1 and anti-ICAM-2 tended to further inhibit PMN transmigration. These results would indicate that in the PMN transmigration response across IL-1 activated endothelium ICAM-1, ICAM-2, and E-selectin each contribute to the total transmigration. At least part of the Mac-1-mediated transendothelial migration appears to be due to interactions with ICAM-1 on the HUVEC under these conditions.

DISCUSSION

The transendothelial migration of PMN is known to be mediated by CD11/CD18 integrins and in particular by LFA-1 and Mac-1, which function in concert, as shown previously [8–10] and in the PMN migration system used here with C5a- or IL-8-induced migration through unstimulated HUVEC or across IL-1-stimulated HUVEC. It is worth noting that the TEM response is certainly preceded by PMN adhesion. However, on resting HUVEC, even during C5a- or IL-8-induced TEM, the adhesion accounts for only 4–7% of added PMN at any given time. The effect of mAb treatments on this low level of adhesion was not reliably measurable. In contrast, the substantial adhesion on IL-1-activated HUVEC (20–30% of PMN), was inhibited by approximately 50% by LFA-1 plus Mac-1 blockade (not shown) [8, 9], whereas migration was blocked by >90%. This indicates that although adhesion must be a prerequisite for migration, there is not a quantitative correlation, probably because migration involves cellular processes in addition to static adhesion.

The results of this study demonstrate that ICAM-1 and ICAM-2 on endothelium both are functionally important counterligands for PMN transendothelial migration. This appears to be the case whether the endothelium is unstimulated or IL-1-activated because combined ICAM-1 plus ICAM-2 blockade had additive inhibitory effect on migration (Figs. 1, 2, and 5). This finding is likely related to the fact that ICAM-2 is expressed at relatively high levels on resting HUVEC, in fact, considerably greater than ICAM-1 [unpublished observations and ref. 18]. However, even after IL-1 activation and increased ICAM-1 expression [5–9], ICAM-2 still appears to have a role as a ligand on HUVEC (Fig. 5). This role appears to be as a ligand for LFA-1, since blockade of LFA-1 on PMN resulted in the same degree of inhibition of PMN transendothelial as blocking of ICAM-1 and ICAM-2 on the HUVEC, at least in the case of unstimulated HUVEC (Figs. 1 and 2). Adding anti-LFA-1-treated PMN to anti-ICAM-1- and anti-ICAM-2-treated HUVEC did not further inhibit migration (Figs. 1 and 2). This is most likely due to blocking of a common pathway or counter-receptor on the PMN and on the HUVEC. The results also demonstrate that the mAb treatments were effective in functionally blocking all LFA-1 on PMN or ICAM-1 or ICAM-2 on the HUVEC.

The studies designed to assess the Mac-1 components of transendothelial migration strongly indicate that, in the case of unstimulated endothelium, ICAM-1 is not an important counter-receptor for Mac-1-mediated migration, even though the Mac-1 mechanism accounts for at least 50% of the total PMN transmigration (Figs. 1, 2, and 3). This is supported by the fact that mAb R6.5 to ICAM-1 had no effect on Mac-1-mediated PMN migration, although it is known to block Mac-1 binding to ICAM-1 [13, 25]. Furthermore, two other mAbs (CBR-IC1/13 and IC1/11) recognizing epitopes in the Mac-1 recognition region [33] also did not inhibit Mac-1-mediated transendothelial migration in response to C5a or IL-8 across unstimulated HUVEC (Fig. 3). Thus, on resting endothelium, the form or level of constitutively expressed ICAM-1 does not appear to participate significantly as a counter-receptor for Mac-1-mediated PMN transendothelial migration in response to chemotactic factors. Recently, similar observations with regard to ICAM-1-independent but Mac-1-mediated PMN migration across platelet monolayers were made [46], confirming that Mac-1 utilizes alternate ligands during PMN migration. In accordance with this, Diamond et al. [47] also presented evidence that unstimulated endothelium expresses a novel ligand for Mac-1-mediated PMN migration.

The experimentation directed at defining the alternate Mac-1-ligand interactions on HUVEC responsible for PMN transendothelial migration indicates that probably none of the well-recognized Mac-1 ligand interactions function as primary counter-receptors on HUVEC mediating this process. An interaction of Mac-1 with either HUVEC bound or synthesized plasma proteins, such as fibrinogen, fibronectin, or other RGD-containing proteins or with other plasma constituents such as Factor X (see text and Fig. 3) or haptoglobulin [6, 27, 40, 44, 45] appears unlikely because none of these components at high concentrations in soluble form altered the Mac-1-mediated transendothelial migration when LFA-1 and ICAM-1 and/or -2 were blocked (Fig. 3 and Fig. 4). Even in the presence of up to 40% human heparinized plasma, IL-8-induced migration was unaffected when LFA-1 and ICAM-1 were blocked (see text). In addition, ICAM-2 also does not appear to contribute to Mac-1-mediated migration, since mAb CBR-IC2/2, which is known to block Mac-1/ICAM-2-mediated adhesion of monocytic cell lines [17], had no effect on Mac-1-mediated PMN transmigration (Fig. 3). It also appears that extracellular matrix protein recognized to mediate PMN adhesion via a Mac-1-dependent mechanism [23, 24, 28], proteins such as fibronectin, collagen (Fig. 3), or laminin (see text) are not the major counterligands in Mac-1-mediated transendothelial migration. Recently, Mac-1 has been recognized as a heparin and glycosaminoglycan (GAG) binding integrin [22, 26]. Because such structures are prominent on endothelium, we investigated their involvement by two approaches. First of all, we added various forms of soluble heparin under conditions shown previously by Diamond et al. to inhibit Mac-1-heparin and heparan sulfate adhesion interactions [22]. Second, GAGs capable of binding to Mac-1 were enzymatically cleaved from HUVEC with the use of heparinase III (or I alone and in combination, not shown) employing conditions shown previously to abolish Mac-1/GAG adhesion [26]. Neither of these treatments altered PMN transendothelial migration (Figs. 3 and 4).

A lectin-binding region of Mac-1, known to recognize yeast β-glucan [38], has received increasing attention because it not only mediates PMN or monocyte activation by phagocytosis of yeast particles or yeast cell walls, but also appears to function in intramembrane association with and possibly signaling for GPI linked membrane proteins including CD16 and CD87 [41–43]. This raised the possibility that the carbohydrate structures on HUVEC might be presented and engaged by Mac-1 on PMN. However, attempts to saturate and compete out such a putative interaction with high concentrations of soluble β-glucan reported to block the β-glucan binding function of Mac-1 on PMN [38], also did not modify PMN transendothelial migration (Figs. 3 and 4). Finally, because Mac-1 has so many putative ligands, which may be expressed on HUVEC or on extracellular matrix (ECM) proteins associated with endothelium, multiple combinations of blockers and antagonists were employed simultaneously such as heparinase III treatment combined with mAb to ICAM-1 and -2, fibrinogen γ-peptide, fibronectin, and β-glucan (Fig. 4) ± soluble laminin and collagen (not shown). However, even these combinations did not modulate Mac-1-mediated transendothelial migration, suggesting that Mac-1 probably engages a yet to be identified ligand on HUVEC during transendothelial migration, rather than utilizing these known ligands as alternates during PMN migration.

Our results indicate that IL-1 activation of HUVEC modifies the ligand(s) available for interacting with Mac-1 for mediating transendothelial migration. Under these conditions, ICAM-1 does appear to contribute to Mac-1-dependent PMN transmigration, as well as serving as a ligand for LFA-1, since mAb R6.5 (anti-ICAM-1) significantly inhibited migration relative to anti-LFA-1 mAb treatment alone (Fig. 5). The shift in the relative role for ICAM-1 under these conditions for Mac-1 engagement may be related to the marked increase in expression of ICAM-1 known to be induced by IL-1 [5, 6, 8, 9, 20, and unpublished observations], but alternative glycosylation of this induced ICAM-1 may also modify Mac-1 recognition as previously proposed [19]. A major role for ICAM-1 in PMN interaction with cytokine-activated HUVEC is in accordance with previous reports [8, 9], although in those studies the contribution of ICAM-2 to the overall transendothelial migration response was not examined. In general, the findings indicate that for transendothelial migration on IL-1-activated HUVEC, the alternative and undefined Mac-1 ligand(s) involved in migration across resting endothelium in response to chemotactic factors are relatively less important. The reason for this might be the up-regulation of ICAM-1 by IL-1, thus providing a sufficient foothold and/or down-modulation of the putative ligand(s) on endothelium by cytokine activation. However, it is also possible that a gradient of a chemotactic factor such as C5a or IL-8 may activate Mac-1 on PMN to a state recognizing a broader range of ligands than occurs on IL-1-activated HUVEC. Evidence for varying affinity states for different ligands has been observed in the case of VLA-4 [48], another integrin capable of recognizing multiple ligands [6, 7]. This is the more likely mechanism because blocking experiments, conducted as in Figures 3 and 4, of Mac-1-mediated PMN migration in response to C5a across IL-1-activated HUVEC yielded results comparable to migration across unstimulated HUVEC (Figs. 3 and 4 and not shown).

The importance of Mac-1 in PMN emigration *in vivo* has recently been questioned, especially since the finding that Mac-1-deficient mice have normal PMN accumulation in the inflamed peritoneum [49]. This suggests that LFA-1 plays a major role in this PMN migration. However, mice genetically deficient in LFA-1 developed 40–50% of the PMN infiltration response in the peritoneum, as compared to wild-type mice [50], suggesting that other CD11/CD18 integrins may also be involved in the LFA-1 knockout mice. Furthermore, in the Mac-1-deficient animals, PMN accumulation in the peritoneum was inhibited substantially more by mAb to LFA-1 (by 78%) than in wild-type mice where the same antibody treatment inhibited PMN accumulation by only 58%. This may be an indication that in Mac-1-deficient animals LFA-1 plays a greater role in mediating a normal PMN infiltration response than in normal mice. Other *in vivo* studies, based on mAb inhibition of PMN migration, have suggested that Mac-1 is an effective alternate in mediating migration into tissues, although this role was only apparent when the function of LFA-1 was blocked. This was observed in the mouse peritoneum, in the rat in dermal inflammation and arthritis, and in dermal and peritoneal inflammation in the rabbit [12, 15, 16, 51]. In most studies, blocking LFA-1 or Mac-1 alone with mAb had little or marginal inhibitory effect, but blocking both of these integrins resulted in dramatic and synergistic inhibition of PMN accumulation in the tissues. It is interesting to note that, in the rabbit, qualitative differences were observed in anti-Mac-1 mAb-treated animals, manifest primarily as diminished PMN migration into the connective tissue with persistent PMN association with the postcapillary venules [16]. Resolution in that study was not sufficient to assess whether the PMN had migrated through the vascular wall or remained trapped in the wall. Thus, overall the weight of evidence would suggest that Mac-1 can function as an effective alternate to the LFA-1 mechanism during *in vivo* PMN emigration and that this is demonstrable *in vitro* by PMN transendothelial migration.

There are some quantitative, rather than qualitative differences between the degree of inhibition *in vitro* by mAbs to LFA-1 and to Mac-1 of human PMN transendothelial and the *in vivo* models of PMN infiltration. These could be species differences or true *in vivo*/*in vitro* system differences. Our results of the degree of inhibition by mAb LPM19C to Mac-1 of PMN transendothelial migration in response to chemotactic factors is somewhat greater than reported by Furie et al. [10]. However, this quantitative difference may be related to differences in endothelial cell culture systems used or, more likely, in the anti-Mac-1 mAb used for blocking the multiple functional interactions of Mac-1 with its ligands. We have screened a large panel of mAbs to human Mac-1 and have observed major differences in inhibition of PMN transendothelial migration, ranging from no inhibition to the degree of inhibition reported here with mAb LPM19C. This mAb has been shown to be particularly effective in blocking at least four different Mac-1 ligand adhesive interactions [25]. We selected this mAb for these reasons and because we have not found it to induce any PMN aggregation or activation as measured by PMN shape

change or oxidative burst induction [unpublished observations], effects that could influence PMN migration.

In conclusion, this study shows that ICAM-2 and ICAM-1 both contribute to PMN transendothelial migration on both resting and cytokine-activated endothelium and that these two ICAMs function in concert as counterligands primarily for LFA-1 in this process. In addition, Mac-1 can mediate PMN transendothelial migration *in vitro* by engaging yet to be defined counter-receptors on endothelium or secreted by endothelium. These are apparently distinct from many of the recognized Mac-1 ligands present in plasma, on ECM proteins, GAGs, and on HUVEC (ICAMs). These results may provide a partial explanation of why inflammatory responses and leukocyte recruitment still can occur in the ICAM-1-deficient transgenic mouse [52, 53]. These findings also would predict that strategies for regulating leukocyte migration *in vivo*, designed to block the ligands on vascular endothelium for CD11/CD18 integrins, will likely be very difficult to develop due to the multiple and redundant interactions.

ACKNOWLEDGMENTS

The authors are grateful to the colleagues mentioned in Materials and Methods who have provided valuable important reagents for these studies. We also gratefully acknowledge the technical help of K. MacLeod and the excellent secretarial assistance of M. Hopkins. This work was supported by grants MT-7684 from the Medical Research Council of Canada and CA31799 from the National Institutes of Health.

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